Final Scientific Report

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BARD Project Number: IS-4267-09

Date of Submission of the report: May 31, 2013

Project Title: Desiccation tolerance in *Salmonella* and its implications

Investigators	**Institutions**
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Co-Principal Investigator (Co-PI): M. McClelland	University of California, Irvine
Collaborating Investigators:	

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
Anhydrobiosis, dehydration, stress response, bioinformatics, systems biology

Abbreviations commonly used in the report, in alphabetical order**:**

DT, dehydration tolerance; *det*, desiccation tolerance gene; LTP, long-term persistence; STm, *S. enterica* sv. Typhimurium

Budget: IS: $159,000 US: $160,000 Total: $319,000

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	1	8	2	11
Submitted, in review, in preparation	1	-	-	1
Invited review papers	-	-	-	-
Book chapters	-		-	
Books	-		-	
Master theses	-	-	1	1
Ph.D. theses	-	-	1	1
Abstracts	-	-	1	1
Not refereed (proceedings, reports, etc.)	2	-	-	2

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	-	1	-	1
Longer Visits (Sabbaticals)	-	-	-	-

Description Cooperation:

Cooperation between the Israeli and the US parties consisted of ongoing discussions through the internet. In addition Dr. Sela has visited Dr. McClelland lab at San-Diego during August 2010 and gave a lecture (acknowledging BARD support) regarding the progress of the project. Dr. Sela met Dr. McClelland and other members of the group. The discussions included analysis of the data obtained at both labs and detailed plans of future experiments. M. McClelland send to S. Sela a collection of *Salmonella* mutants to be screen for desiccation-sensitive mutants as well as costumed-made slides containing *Salmonella* DNA-microarray. These DNA microarrays were used in S. Sela lab to identify *Salmonella* genes, which are upregulated during dehydration. M. McClelland also assisted in the bioinformatics analysis of the data. These widely useful tools are in active use in this and related project with acknowledgement of BARD. This cooperation has culminated in a joint paper published in the journal of "Applied and Environmental Microbiology" (Gruzdev, M., McClelland, M., Porwollik, S., Ofaim, S., Pinto, R., and Sela-Saldinger, S. (2012). Global transcriptional analysis of dehydrated *Salmonella enterica* serovar Typhimurium. App. Env. Microbiol. 78:7866-7875.).

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Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	-	-	-	-
Issued (allowed)	-	-	-	-
Licensed	-	-	-	-

Abstract

Salmonella enterica is a worldwide food-borne pathogen, which regularly causes large outbreaks of food poisoning. Recent outbreaks linked to consumption of contaminated foods with low water-activity, have raised interest in understanding the factors that control fitness of this pathogen to dry environment. Consequently, the general objective of this study was to extend our knowledge on desiccation tolerance and long-term persistence of *Salmonella.* We discovered that dehydrated STm entered into a viable-but-nonculturable state, and that addition of chloramphenicol reduced bacterial survival. This finding implied that adaptation to desiccation stress requires *de-novo* protein synthesis. We also discovered that dried STm cells develop cross-tolerance to multiple stresses that the pathogen might encounter in the agriculture/food environment, such as high or low temperatures, salt, and various disinfectants. These findings have important implications for food safety because they demonstrate the limitations of chemical and physical treatments currently utilized by the food industry to completely inactivate *Salmonella*. In order to identify genes involved in desiccation stress tolerance, we employed transcriptomic analysis of dehydrated and wet cells and direct screening of knock-out mutant and transposon libraries. Transcriptomic analysis revealed that dehydration induced expression of ninety genes and down-regulated seven. Ribosomal structural genes represented the most abundant functional group with a relatively higher transcription during dehydration. Other large classes of induced functional groups included genes involved in amino acid metabolism, energy production, ion transport, transcription, and stress response. Initial genetic analysis of a number of up-regulated genes was carried out). It was found that mutations in *rpo*S, *yah*O, *ace*A, *nif*U, *rpo*E, *ddg, fnr* and *kdp*E significantly compromised desiccation tolerance, supporting their role in desiccation stress response.

Evaluation of the research achievements

Salmonella enterica is a worldwide food-borne pathogen, which regularly causes large outbreaks of food poisoning. Recent outbreaks linked to consumption of contaminated foods with low water-activity, have raised interest in understanding the factors that control fitness of this pathogen to dry environment. Consequently, the general objective of this study was to extend our knowledge on desiccation tolerance and long-term persistence of *Salmonella.*

The specific objectives were as follows: (1) Expand the knowledge on *Salmonella* anhydrobiosis and characterize environmental factors that might affect desiccation and long term survival in the air-dried state; (2) Examine the effect of desiccation on tolerance to other stressors (cross-tolerance), such as temperature, osmolarity, pH, UV-irradiation and disinfection agents; (3) Determine the effect of desiccation on virulence in a mouse model of oral infection; (4) Identify candidate genes important for desiccation tolerance and long-term persistence: (5) Functionally characterize desiccation-tolerance genes in various food-related models.

We have met most of the objectives, and a brief summary of the major results is listed here. We performed an initial characterization of environmental factors that affect the tolerance of *S. enterica* serovar Typhimurium (STm) to desiccation. We discovered that dehydrated STm entered into a viable-but-nonculturable state, and that addition of chloramphenicol reduced bacterial survival. This finding implied that adaptation to desiccation stress requires *de-novo* protein synthesis (Gruzdev et al., 2012a). We also discovered that dried STm cells develop cross-tolerance to multiple stresses that the pathogen might encounter in the agriculture/food environment, such as high or low temperatures, salt, and various disinfectants (Gruzdev et al., 2011). These findings have important implications for food safety because they demonstrate the limitations of chemical and physical treatments currently utilized by the food industry to completely inactivate *Salmonella*. In order to identify genes involved in desiccation stress tolerance, we employed two approaches: (a), transcriptomic analysis of dehydrated and wet cells using microarrays; and (b), direct screening of knock-out mutant and transposon libraries. The initial characterization of the insertion positions in the transposon library constructed for this project is now published (Canals et al., BMC Genomics, 2012). The ongoing results of the screens are presented in tables later in the report. Transcriptomic analysis revealed that dehydration induced expression of ninety genes and down-regulated seven. Ribosomal structural genes represented the most abundant functional group with a relatively higher transcription during dehydration. Other large classes of induced functional

groups included genes involved in amino acid metabolism, energy production, ion transport, transcription, and stress response. Initial genetic analysis of a number of up-regulated genes was carried out and revealed the involvement of *ace*A, *nif*U, *rpo*E, *ddg*, *fnr* and *kdp*E in the *Salmonella* desiccation stress response (Gruzdev et al., 2012b).

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1. Effect of environmental factors on desiccation tolerance and long-term persistence

1.1 Effect of osmolarity

Effect of osmolarity on desiccation tolerance (DT) and long-term persistence (LTP) was examined by comparing STm desiccation in double-distilled water (DDW; hypotonic solution), phosphate buffered saline (PBS-pH 7.2) and increasing concentration of NaCl (Fig. 1). No significant difference ($P>0.05$) in DT or LTP was found between cells desiccated in the presence of PBS and (DDW). Increasing the osmotic strength to 0.125 M NaCl resulted in higher mortality during desiccation and decreased the LTP at 4ºC. While cells resuspended in DDW and PBS maintained their population during 12 weeks, the cells resuspended in 0.125-1M NaCl reached undetectable level after 8-12 weeks of storage.

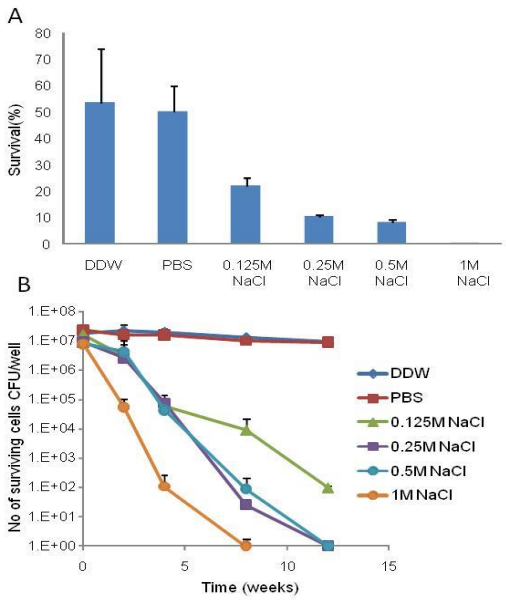

Figure 1. Effect of osmolarity on DT and LTP. *Salmonella* cells were grown for 20 h at 37ºC in LB plate. Cells were washed three times in DDW, PBS or NaCl (0.125, 0.25, 0.5 or 1 M) and then were resuspended in the indicated solutions to the final concentration of $2x10^9$ CFU/ml. 50µl aliquots were put into wells of 96-wells polystyrene plate. The plate was dried in a Biosafety hood for 22 h at 25ºC, and then stored for 12 weeks at 4ºC. Viable counts were determined immediately after dehydration and after 2, 4, 8, and 12 weeks of storage. DT (A) is presented as the average percentage of surviving cells following desiccation (±SD) and LTP (B) is presented as the mean viable count (±SD) from at least two independent experiments, each performed in triplicate.

1.2 Effect of pH

Desiccation of STm was performed under increasing pH values (Fig. 2). Desiccation at acidic conditions resulted in decreased DT (A) and LTP (B), compared to desiccation at high pH values. In fact, cells incubated at pH 3 reached undetectable levels after 22 h of desiccation. In contrast, cells incubated at pH 11 survived the desiccation with 4-log CFU decrease. Cells

desiccated at pH 4 and 5 demonstrated ~ 6 log reduction within 12 weeks of storage at 4°C, while cells desiccated at basic environment, except pH 11 maintained their original population.

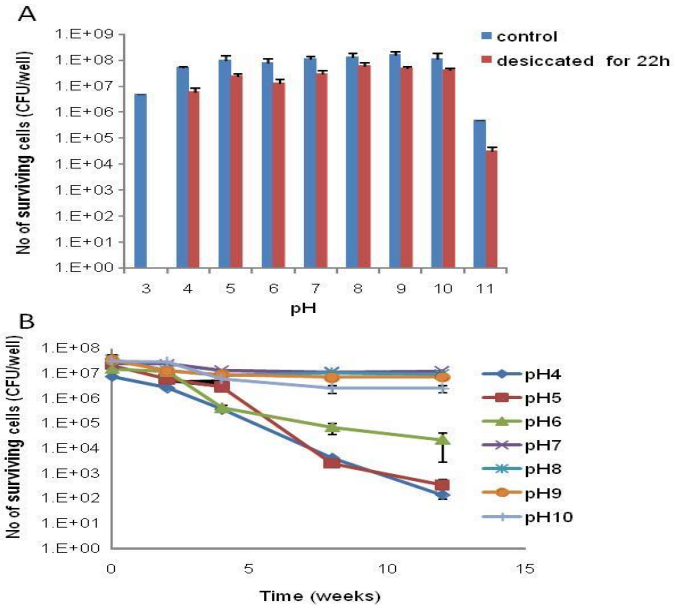

Figure 2. Effect of pH on DT (A) and LTP (B). Bacterial cells were prepared as described in Figure 1. Prior to desiccation cells were resuspended in DDW adjusted to pH 3-11 with 1M HCl for acidic solutions and with 1M NaOH for basic conditions. Desiccation experiments were performed as described above. Cells incubated for 22 h in DDW (without desiccation) served as a control. Average viable counts (±SD) from three independent experiments are presented.

1.3 Effect of duration of desiccation process

Effect of the time of dehydration on DT and LTP was also determined (Fig. 3). Bacterial cells (10^8 CFU) suspended in 15, 25 and 50 µl were dried until no water was visually apparent (6.5, 11 and 22 h, respectively). Shorter desiccation time has reduced both DT (3A) and LTP (3B) scores compared to cells dehydrated in 50 µl (22 hours to full dehydration).

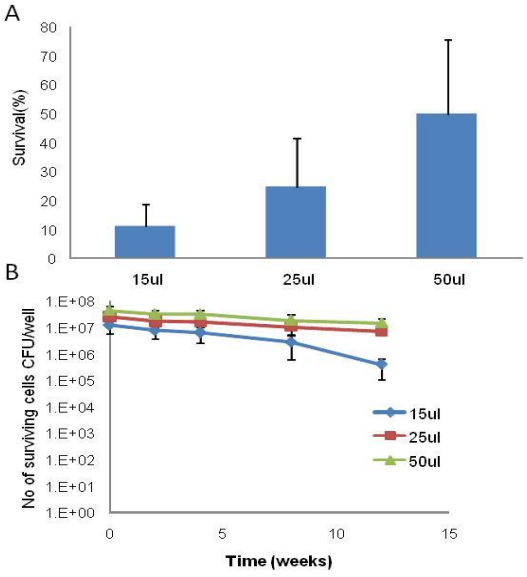

Figure 3. Effect of dehydration duration on DT and LTP. Bacterial cells were prepared as described in Figure 1. An inoculum of 10^8 CFU/well in 15, 25 and 50 µl aliquots was desiccates in 96-well plate for 6.5, 11 and 22 hours respectively to full dehydration. DT is presented as the average percentage of surviving cells (±SD) (A), and LTP is presented as the mean viable count (±SD) (B) from three independent experiments, each performed in triplicate.

1.4 Effect of nutrients and compatible solutes

Effect of exogenous nutrients and compatible solutes on DT and LTP was tested (Fig. 4) STm cells dried in LB medium and in 100mM trehalose or sucrose, demonstrated significantly higher DT compared to cells desiccated in

DDW. In contrast, addition of 100 mM of glycine-betaine to the drying cells, didn't affect DT (4A). Drying in trehalose or sucrose solution, but not glycine-betaine or LB, has increased LTP (4B).

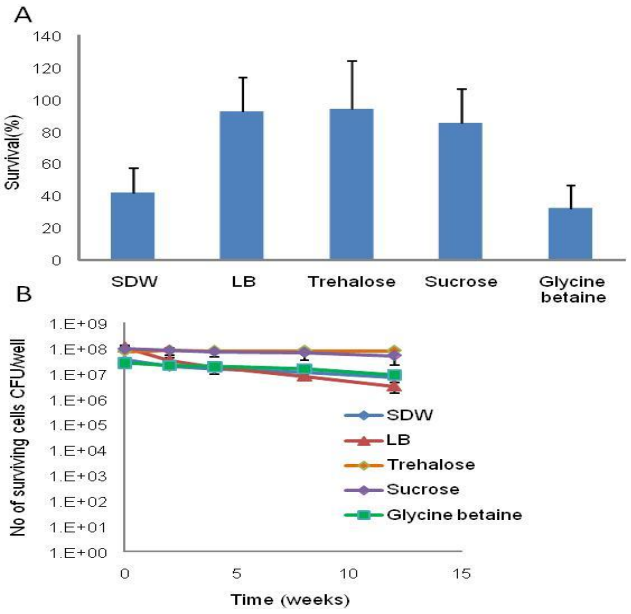

Figure 4. Effect of compatible solutes and nutrients on DT and LTP. Bacterial cells were prepared as described in Figure 1. Prior to desiccation bacteria were resuspended in SDW, LB broth, 100 mM trehalose, sucrose or glycine betaine. Desiccation experiments were performed as described above. DT is presented as the average percentage of surviving cells (±SD) (A), and LTP is presented as the mean viable count (±SD) (B) from two independent experiments, each performed in triplicate.

1.5 Effect of growth-phase

Effect of bacterial growth-phase on DT and LTP was also tested (Fig. 5). Cells derived from logarithmic growth phase (4 h) were more sensitive to desiccation (A), and exhibited lower DT and LTP (B) compared to cells derived from stationary growth phase (20 h). No significant difference was found between stationary cells grown in liquid (LB broth) or on solid media (LB agar).

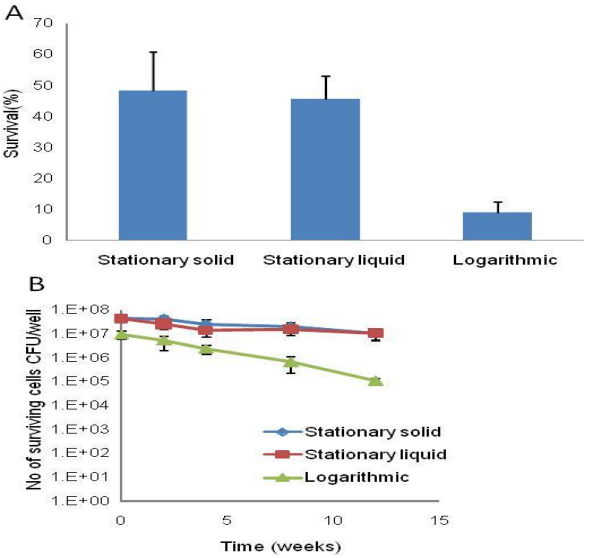

Figure 5. Effect of growth-phase on DT and LTP. Cells derived from different growth phases were collected, washed three times in SDW and brought to a final concentration of 2x109 CFU/ml. Desiccation experiments were performed as described above. DT is presented as the average percentage of surviving cells (±SD) (A), and LTP is presented as the mean viable count (±SD) (B) from three independent experiments, each performed in triplicate.

2. Examination of the effect of desiccation on tolerance to other stressors

2.1 Effect of exposure to NaCl and bile salts

Salmonella was exposed to increasing concentrations of NaCl and bile salts for 2 h (Fig. 6). Higher DT was observed in desiccated- compared to non-desiccated cells. Furthermore, desiccated cells were able to maintain their original numbers in 1-5 % bile salts and 0.1-0.5 M NaCl, while the number of the control (non-desiccated) cells continuously declined in a dose-dependent manner.

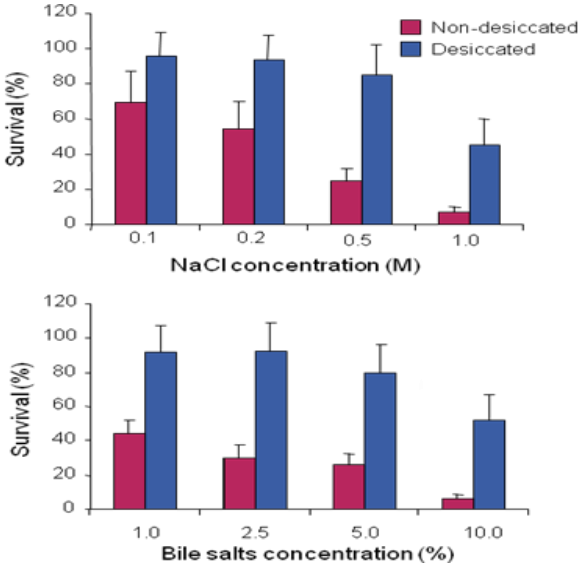

Figure 6. Survival of *S.* Typhimurium following exposure to increasing concentrations of NaCl (A) and bile salts (B). Bacteria were treated and desiccated as described previously. Non-desiccated cells were incubated for 22 h in SDW at 25ºC. Bacterial cells were exposed to 0.1-1M of NaCl and to 1-5% of bile salts (oxgall) for 2 h. The bars represent the average DT values (+SD) in three independent experiments, each performed in triplicate.

2.2 Effect of exposure to disinfecting agents

Salmonella cells were exposed to increasing concentrations of ethanol, hydrogen peroxide, sodium hypochlorite and quaternary ammonium-chloride (DDAC) for the indicated times (Fig. 7).

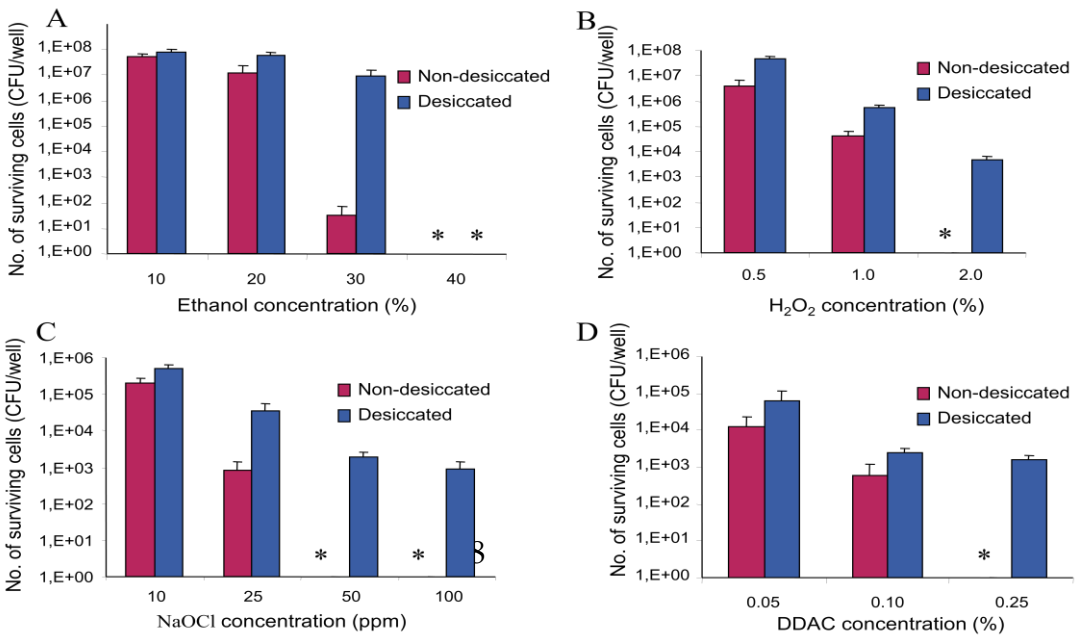

Figure 7. Effect of disinfecting agents on the survival of desiccated and non-desiccated *Salmonella*. Desiccated and non-desiccated cells were treated as described for figure 6. Afterwards bacteria were exposed to different concentrations of ethanol for 5 min (A), hydrogen peroxide for 30 min (B), sodium hypochlorite for 10 min (C) and DDAC for 5 min (D). The average numbers (+SD) of surviving cells from at least two independent experiments are presented. Asterisk denotes *Salmonella* counts below detection limit (H_2O_2 and ethanol < 100 CFU, NaHOCl3 and DDAC < 30 CFU).

Desiccated cells demonstrated significantly higher tolerance to all the disinfectants. Moreover, desiccated cells still survived under conditions where non-desiccated cells were undetectable, such as exposure to 2% hydrogen peroxide for 30 min, 50-100 ppm sodium hypochlorite for 5 min, and 0.25% DDAC for 5 min. Desiccated cells survived exposure to 30% ethanol for 5 min with only 1.0 log reduction, compared to 6.5-log decrease in non-desiccated cells. However, both populations' numbers declined below the detection limit (100 CFU) following exposure to 40% ethanol for 5 min.

2.3 Effect of exposure to organic acids

In contrast to all other stressors, exposure of *Salmonella* to acetic- or citric-acid at pH 3.0 had an opposite effect on desiccated cells. In both cases, desiccated cells were more susceptible to the acidic conditions compared to non-desiccated cells (Fig. 8).

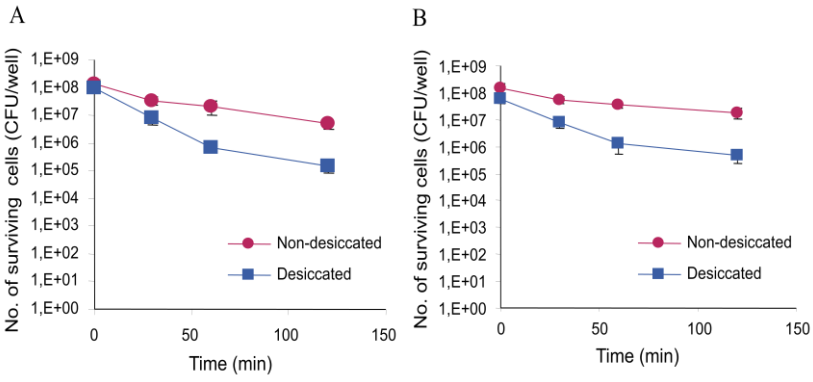

Figure 8. Effect of organic acids on survival of desiccated and non-desiccated S. Typhimurium. The cells were treated as described for figure 6 and then exposed for 30-120 min to 50 mM citric acid (A) or 80 mM acetic acid (B), both at pH 3.0. The average numbers (+SD) of surviving cells from at least two independent experiments are presented.

2.4 Effect of exposure to UV irradiation

Exposure to UV irradiation (125 µW/cm2) for 25 minutes resulted in complete eradication of non-desiccated cells, compared 3-log reduction in desiccated cells (Fig. 9).

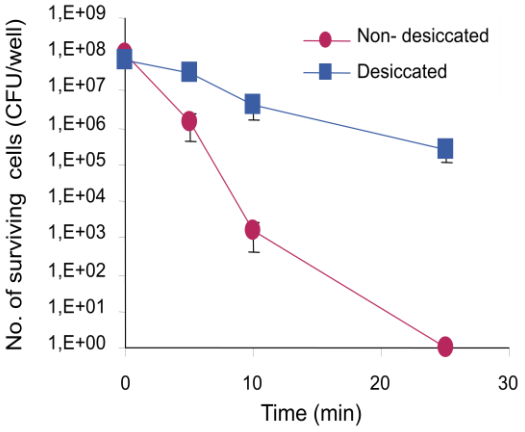

Figure 9. Effect of UV irradiation on survival of desiccated and non-desiccated S. Typhimurium. The

cells were exposed for 5-25 min to a UV irradiation (125 µW/cm2). The average numbers (+SD) of surviving cells from at least two independent experiments are presented.

2.5 Effect of exposure to dry heat

Desiccated cells have demonstrated high tolerance to 1 h exposure to dry heat with apparently no change in the viable count at 60 ºC, and 1.5- and 3.1-log reduction at 80 and 100ºC, respectively. In contrast, non-desiccated cells were highly suceptible, with as much as 3 log reduction at 60 ºC, and 8-log reduction (under detection limit) at 80 and 100 ºC. To examine if desiccated bacteria could still maintain heat tolerance after rehydration, desiccated cells were rehydrated with SDW immediately before exposure to heat. No significant difference was found between rehydrated and non-desiccated bacteria (Fig. 10A).

Since exposure to 80 and 100 ºC for 1 h resulted in complete eradication of non-desiccated *Salmonella*, we have also investigated the killing kinetics at 100 ºC. While, non-desiccated cells were completely inactivated within 10 min, and rehydrated cells were killed after 20 min, the desiccated cells remained viable with only a 3-log CFU reduction at 60 min (Fig. 10B).

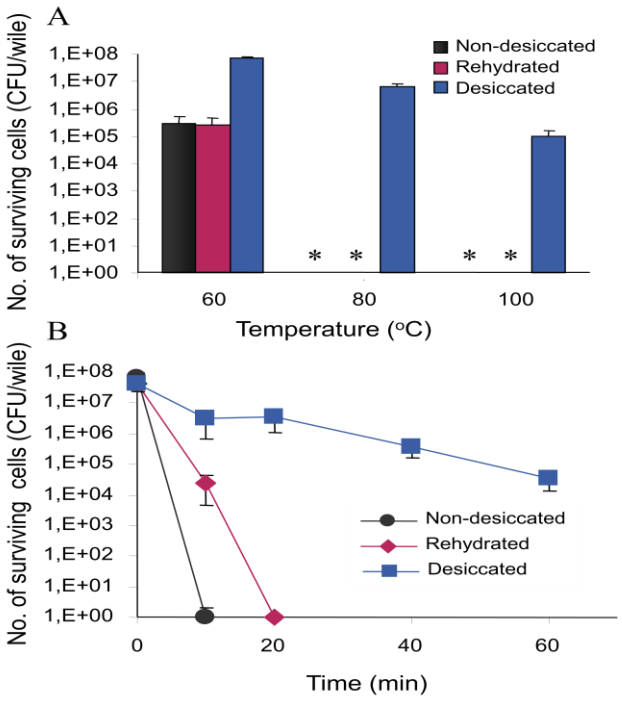

Figure 10. Effect of desiccation on thermal tolerance of *Salmonella*. Desiccated, non-desiccated and rehydrated cells were exposed to temperatures of 60, 80 and 100ºC for 1 h (A), or to 100ºC for up to 60 min (B). The average numbers of surviving cells (+SD) from at least two independent experiments are presented. Asterisk denotes *Salmonella* counts below detection limit (< 10 CFU).

2.6. Effect of desiccation on cross-tolerance in other *Salmonella* serotypes

To examine wether the cross-tolerance phenomenon is unique to S. Typhimurium strain SL1344, the response of serotypes Enteritidis, Hadar, Infantis, and Newport to the same stressors was tested (Table 1). Similar to *S.* Typhimurium, desiccation significantly ($p < 0.01$) enhanced the tolerance of all four serotypes to NaCl (1M, 2 h) bile salts (10%, 2 h), ethanol (30%, 5 min), dry heat (100ºC, 1 h) and UV irradiation (125µW/cm^2, 25 min). Except for

serotype Hadar, all desiccated strains survived exposure to sodium hypochlorite (100 ppm, 5 min), hydrogen peroxide (2%, 5 min) and DDAC (0.25 %, 5 min) with 4-6 log CFU reduction, while non- desiccated cells reached undetectable levels (>7.0 log CFU reduction) under these conditions. Exposure of all strains to citric acid (50 mM, pH 3.0) resulted in significantly ($p < 0.01$) higher inactivation (0.6-2.3 log CFU reduction) in desiccated cells compared to non-desiccated bacteria (0.2-0.6 log reduction), as was previously demonstrated for *S.* Typhimurium.

3. Identification of candidate genes important for desiccation tolerance and long-term persistence

3.1 RIVET

3.1.1 Identification of desiccation-induced genes

STm RIVET library, previously constructed in our lab, was used to screen for candidate genes (promoters) involved in desiccation tolerance. The RIVET library was pooled and 10^8 clones per well were air-dried in triplicates, as described above. Following rehydration and resuspension, bacteria were plated on LB+10% sucrose to identify potential promoters which resulted in *tnpR* activation and the loss of the *res1*-cassette containing the *neo* and *sacB* genes, encoding for kanamycin resistance gene and levansucrase (Merrell and Camilli, 2000). Following verification of the loss of Kan resistance, chromosomal regions near the 5' of the *tnpR* gene were identified as described (Caetano-Anollés et al., 1991) and sequenced. Bioinformatic analysis was used to identify regions of potential promoters located upstream of the *tnpR* insertion. A list of genes, whose putative promoters were potentially activated during desiccation, is presented in Table S1.

3.1.2. Functional analysis of selected genes

Several regulatory and structural genes identified by RIVET were functionally analyzed. Site-specific deletion mutations were generated in genes *pflC, envZ, mdoH, barA* and the fate of *Salmonella* wt and mutant strains was tested using the DT and LTP assays (Fig. 11 A,B). Mutants in genes *yfh*A and *bar*A were significantly ($p<0.05$) hampered in DT with around 10% survival (compared to the wt). Similarly the two mutants were also defective in LTP, with 2 log CFU reduction in Δ*barA* mutant and >7 log CFU reduction in the Δ*yfhA* mutant after 2 weeks of storage at 4^{o}C. In contrast, the wt strain survived well with less than1 log CFU reduction.

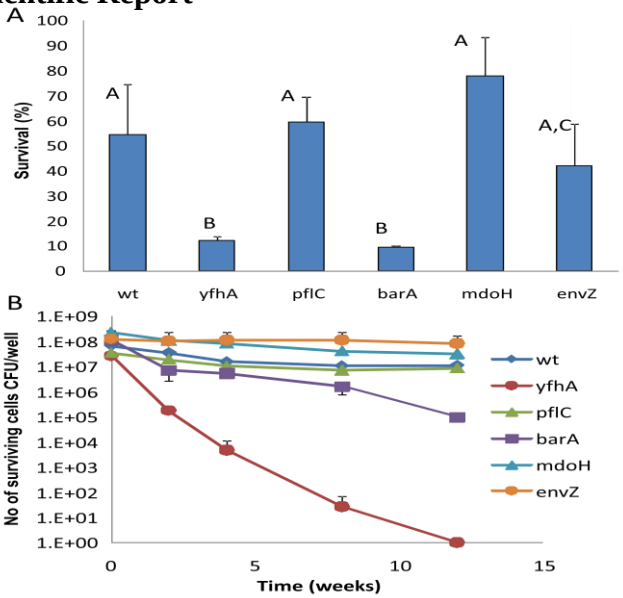

Figure 11. Functional characterization of selected mutants in genes identified by RIVET. Mutants and wt were treated and desiccated as described previously. The average percentage of surviving cells (±SD) for DT (A) and the mean viable counts (±SD) for LTP (B) from three independent experiments are presented. Different letters indicate significant differences ($p<0.05$) in survival percentages.

3.2 Screening of ordered-mutations' library

A partial ordered STm library (Kan insertions) containing 1036 individual mutations mostly in genes unique to the *Salmonella* genus, was dried in 20% sucrose in Dr. McClelland lab and sent to Dr. Sela. The dried mutants were resuscitated by re-suspension in 100 µl LB broth and incubated at 37 C in an ELISA plate reader. Optical density at 595 nm (OD_{595}) was recorded every 1 h. Mutants which has a longer lag time compared to the WT, but reached the final WT OD_{595} at 16 h incubation, were assumed to be putative *det* mutants. 109 such mutants were identified and 25 of them were individually tested and showed decreased desiccation tolerance compared to the WT strain. The identity of these mutants was verified using specific primers and DNA sequencing. The designated mutations were confirmed in 14 mutants (60%) (Table S2). Phage P22 transduction experiments were performed to verify the association between the specific mutations and the DT phenotype. Out of 14 selected transductants that came out by the screening, desiccation-compromised phenotype was confirmed in only two mutants, i.e. *rpo*S and *yah*O (Fig. 12A). These mutants were tested for DT and LTP at 4^{o}C and the results are illustrated in Fig. 12B. The *rpoS* deletion mutant was highly impaired in both DT and LTP. This mutant demonstrated very low survival (~0.1%) and reached undetectable levels, as early as 4 weeks of cold storage. The second mutant (*yahO*) was moderately compromised in DT and LTP compared to the WT.

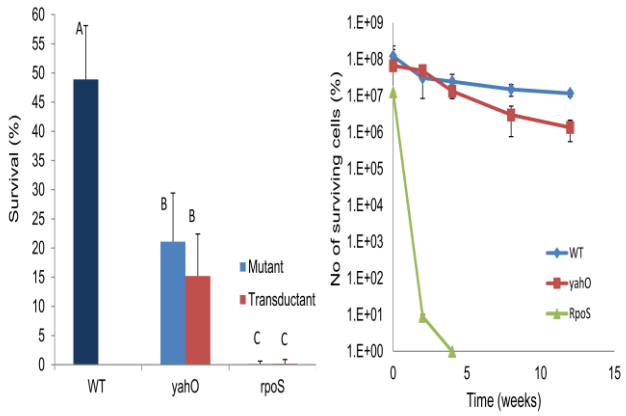

Figure 12. Functional characterization of selected mutants in genes identified by ordered-mutations' library. Mutants, P22 transductants and wt strains were treated and desiccated as described previously. The average percentage of surviving cells (±SD) for DT (A) and the mean viable counts (±SD) for LTP (B) from three independent experiments are presented. Different letters indicate significant differences ($p<0.05$) in survival percentages.

3.3 Identification of candidate *det* genes by microarray

Since, the complete ordered mutation library was not ready at the time, McClelland has provided custom-made microarray slides of STm genome in order to facilitate the identification of potential *det* genes. Total RNA from desiccated and non-desiccated (in SDW) cells incubated for 22h at 25^{o}C). RNA was isolated, labeled and hybridized to the *Salmonella* ORF microarray- chips STv7E at the MicroArray core facility (Faculty of Medicine, Ein Kerem, The Hebrew University, Jerusalem). The microarray study was performed in 4 independent (biological) experiments. The data was analyzed using LIMMA software package. The software provide the statistically significance of differentially expressed genes based on all the arrays on a specific experiment. Results with signal to noise ratio ≥ 1.8 ($\log_2$ FC ≥0.85) with $P < 0.05$ were considered significant.

Ninety three up-regulated and 7 down-regulated genes were identified. These genes were further analyzed using Blast2GO (Conesa et al., 2005) tool. General functions of differentially expressed genes ($\log_2$ FC order) are summarized in Table S3.

3.3.1 Bioinformatics analysis of positively regulated genes identified by microarray

The identified genes were analyzed using Blast2GO tool (Conesa et al., 2005) and classified by function, cellular process or cell fracture. (Fig. 13) The largest number (21) of up-regulated genes found to be involved in ribosome structure and biogenesis (Fig 7A) as well as in amino acids transport and metabolism (17). Most of the tested genes has protein (31) and RNA (21) binding activity Fig. 7B). In addition, most of the genes are found to be membrane associated (Fig 13C).

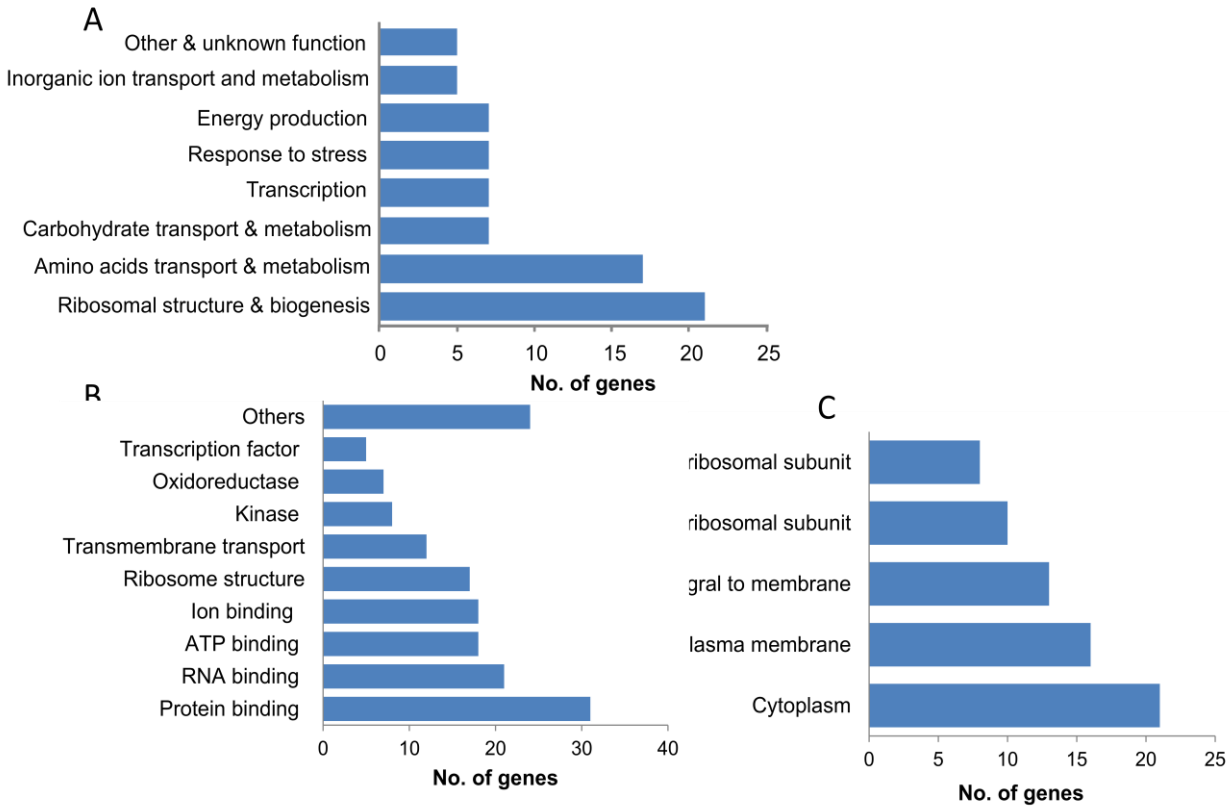

Figure 13. Bioinformatic analysis of up-regulated genes was performed using Blast2GO (Conesa et al., 2005) tool (http://www.blast2go.com/b2ghome). The genes were classified by function (A), cellular process (B) or cellular fracture (C).

3.3.2 Analysis of up-regulated operones

Up-regulated genes were analyzed with MicrobesOnline Operon Prediction tool (Price et al., 2005) on (http://www.microbesonline.org/operons/). In general, the up-regulated genes belong to 9 diferent operons involved in pottasium transport, histidine, glutamate, dicarboxylate and glycerophospholipid methabolism, nitrogen fixation, ribosome structure and transcription (Figure S1).

3.3.3 Functional analysis of selected genes identified by microarray

From each operon, we selected one gene with the highest fold-change and generated mutations by the λ Red Recombinase method (Datsenko and Wanner, 2000). Since *kdp* operon had the highest fold-change, we decided to generate a mutation also in its regulatory gene, *kdp*E, although it wasn't detected by the microarray screen. We also generated mutants in the regulatory gene *fnr*, a universal stress protein UspE (*yda*A), as well as in lipid A biosynthesis palmitoleoyl acyltransferase (*ddg*). These mutants were tested for DT and LTP (Fig. 14). Mutants in genes *ace*A, *nif*U, *rpo*E, *ddg*, *fnr* and *kdp*E demonstrated significantly

(P<0.05) lower DT compared to WT (Fig. 14A). The *kdpE* mutant had the lowest DT (1.82±1.3%) compared to the WT (49.9±9.4%). This strain had also the lowest persistence at 4ºC (LTP) reaching undetectable levels after 4 weeks of storage. Four out the five other deletion mutants with lower DT also displayed lower LTP during cold storage with 2-3 log CFU reduction compared to ~1 log reduction in the WT strain (Fig 14B).

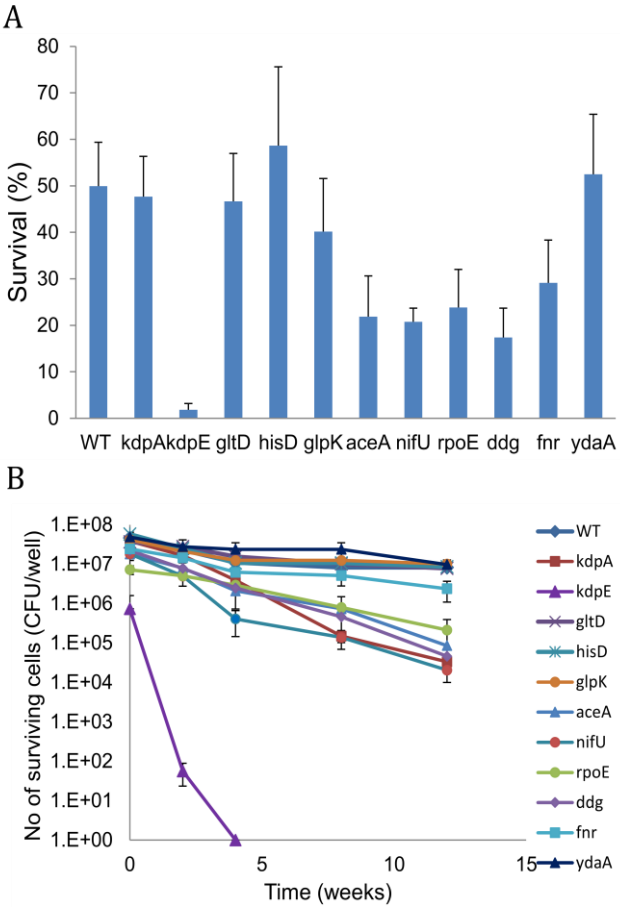

Figure 14. Functional characterization of selected mutants in genes identified by microarray. Mutants and wt were treated and desiccated as described previously. The average percentage of surviving cells (±SD) for DT (A) and the mean viable counts (±SD) for LTP (B) from three independent experiments are presented. Different letters indicate significant differences (p<0.05) in survival percentages.

Multiple genes of the histidine and arginine biosynthetic pathways were also induced during dehydration, which perhaps indicates the involvement of the two amino-acids in the adaptation of *Salmonella* to desiccation. However, neither deletion of the *his*GI operon, (histidine biosynthesis), nor deletion of the *arg*BC operon (synthesis of arginine), affected bacterial survival (Fig.15AB), inferring that histidine and arginine are apparently individually dispensable for survival under dehydration. However, a double mutant Δ*his*GI/*arg*BC was compromised both in dehydration tolerance (Fig. 15A) and the long-term persistence (Fig. 15B). Biosynthesis pathways for histidine and arginine are controlled by the *argR* repressor. Although deletion of *arg*R gene did not affect DT (Fig. 15C) it significantly decreased LTP. The exact role of these two amino acids in desiccation tolerance remain to be studied.

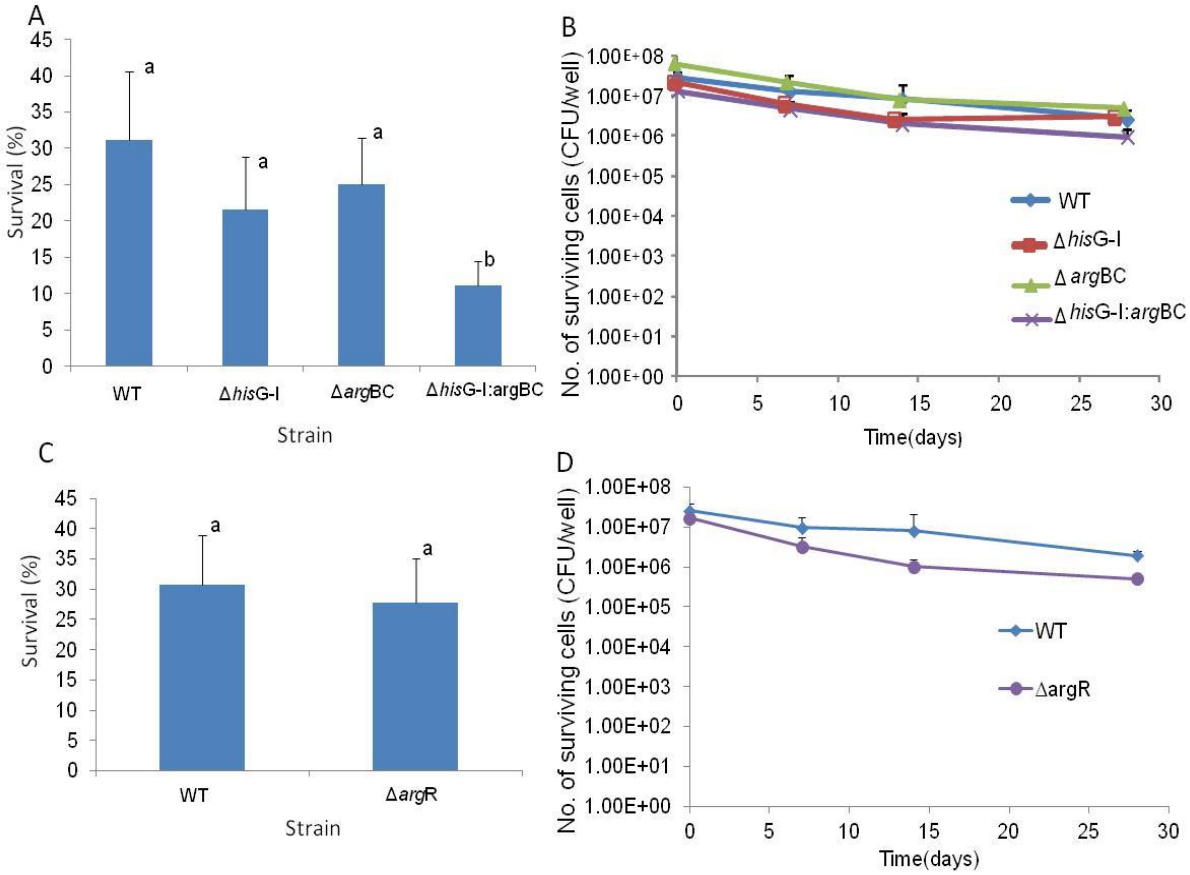

Figure 15. The role of arginine and histidine operons in DT and LTP. A-B: Functional characterization of operons *arg*BC and *his*GI. C-D: functional characterization of ArgR regulator. Mutants and wt were treated and desiccated as described previously. The average percentage of surviving cells (±SD) for DT (A, C) and the mean viable counts (±SD) for LTP (B, D) from three independent experiments are presented. Different letters indicate significant differences ($p<0.05$) in survival percentages.

Among the dehydration-induced genes, the highest up-regulation was observed in the *kdp*ABC genes. This operon encodes a high affinity K^+-uptake system in many bacteria. We demonstrated that mutation in operon synthesis activator KdpE, results in extremely impaired DT and LTP phenotype. However, deletion of *kdp*ABC operon encoding to proteins responsible to potassium transporting channel structure and assembly, didn't affect survival phenotype (Fig. 16A,B) indicating that involvement of KdpE in desiccation stress response may occur via activation of factors other than potassium uptake system,

4. Study the effect of desiccation on *Salmonella* virulence in mice

We first focused on the evaluation of the optimal feeding methodology for the mice experiments with dried feed. Following literature searchs and our own findings regarding cross-tolerance to other stress, we decided that the dried *Salmonella* cells would be resuspended in sterile double-distilled water and immediately introduce into the mouse by gavage feeding.

Next, we determined the infection efficiency of orally infected mice. The efficiencly varied over three orders of magnitude. This fact precluded the inidvidual measurement of dry versus wet bacteria because reliable results would require a very large number of mice. Thus, we instead compared a mxture of wet versus dry bacteria, together. We prepared Salmonella in 96 well plates by drying in the mediun and condititions described earlier. We prepared plates for three available antibiotic resistances; kanamycin, chloramphenicol, streptomycin, and tetracycline resistance. Each plate was resuspended with freshly grown washed pellet from actively growing bacteria in every pairwise combination and assessed for ratio in vitro. This experimental design ensured that any variation due to differential fitness of strains was controlled.

Next, we performed experiments by gavage in mice and recovered bacteria in the gut and spleen. In control mouse experiment, the standard deviation of counts greatly exceeded the average ratio of antibiotic resistance markers, indicating that the test needed an improvement in power.

We thus designed an alternative strategy, which is ongoing. We inserted different 18 base bar codes into a neutral location in the genome, generating hundreds of clones each with a different barcode. We plan to use these mutants in different mixtures, each treated in a different manners to monitor population complexity and thus infection efficiency. Thus, for example, we could split half of the barcode clones into a dessication class and half into a wet growth class, mix them and then monitor the hundreds of barcodes, simultaneously. This method, by avoiding the use of only two markers per mouse, will be highly tolerant of the vast differences (bottlenecks) we have observed in oral infection. It will also allow us to reduce the dose below the very high, perhaps non-physiological dose, currently used for oral infection. In addition, the strategy holds out the prospect that we could combine a large number of different stress treatments into a *single* side-by-side measure in the same mouse, relying on a different set of barcodes for each pre-stress.

5. Additional resources developed over the course of the project.

During the course of the project an additional over 4000 single gene deletion mutants in Typhimurium were made, representing Kanamycin and/or chloramphenicol resistance markers in essentially all genes that can sustain a mutation when growing in Luria Broth. The preliminary quality control analysis of these genes has led to a number of papers. We also have constructed additional transposon libraries in some of the serovars mentioned earlier, in which comparative stress responses may be critical to understanding relative risk. These include Enteritidis and Newport. In related studies, inspired by the need to understand diversity in stress response in the BARD project, we have sequenced additional strains of Enteritidis, Hadar, Infantis, and Newport. Sequence variation between strains may eventually be correlated with stress phenotypes. Finally, the approaches and bioinformatics generated in this project were used in a number of other publications and, accordingly, support from BARD is acknowledged.

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Supplements

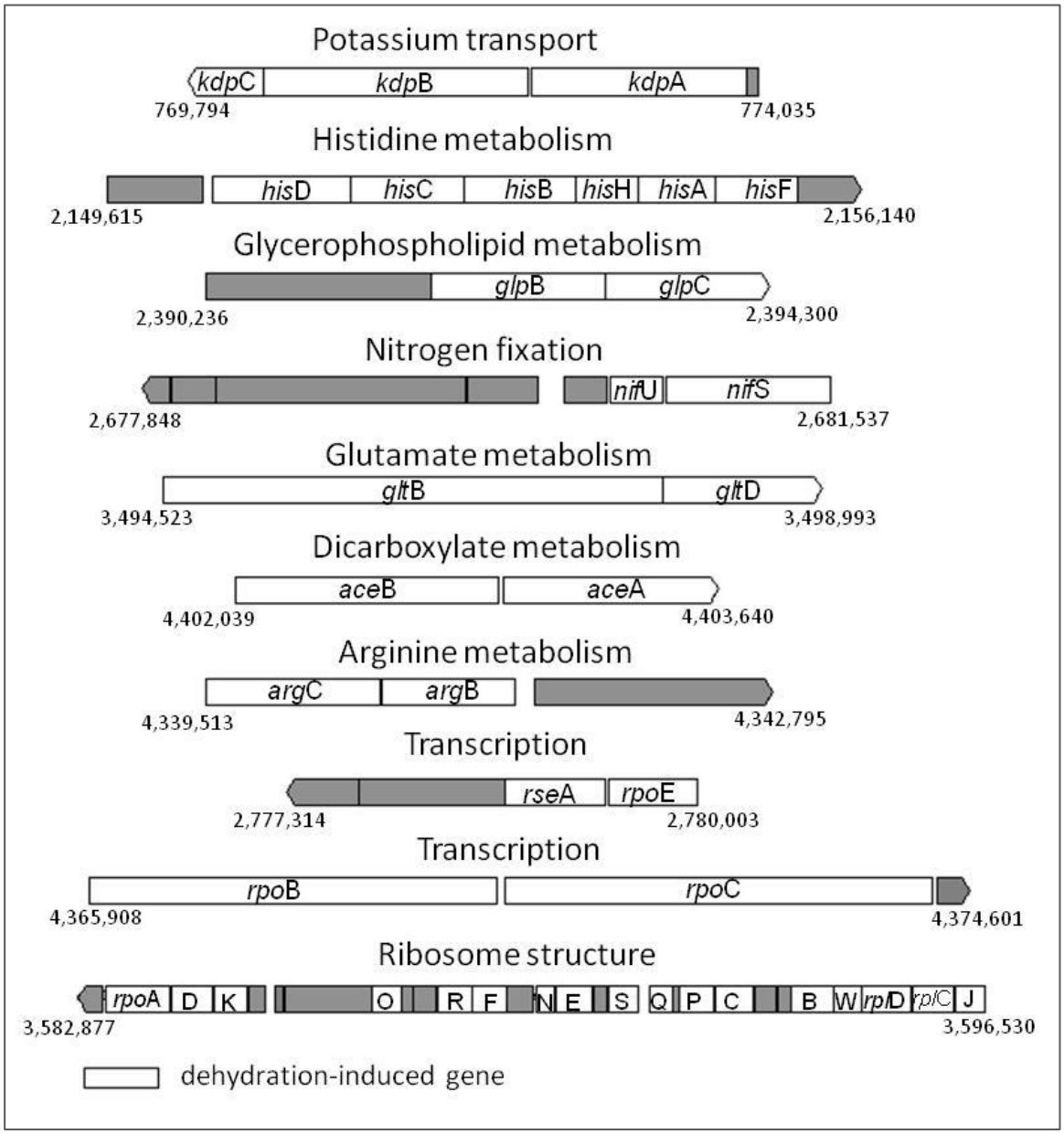

Figure S1. Bioinformatic analysis of up-regulated genes was performed MicrobesOnline Operon Prediction (Price et al., 2005) tool. (http://www.microbesonline.org/operons/) and the genes were divided into operons. The letters in ribosomal structure operon denotes: K-rpsK; O-rplO; R-rplR; N-rpsN; E-rplE; lN-rplN; Q-rpsQ; P-rplP; W-rplW; J-rpsJ.

Table S1. A list of putative desiccation-tolerance (*det)* genes identified by RIVET**.**

General function	Gene
Signal transduction	*yfh*G, *yfh*A, *env*Z, *yoj*N, *bar*A, *cst*A
Energy production and conversion	*suc*A, *acn*B, *hyb*C, *dsb*D, *cyd*B, *ast*D, *adh*E, *nuo*G, *mae*B, *yfi*Q, glpK
Carbohydrate transport and metabolism	*glg*B, *pts*A, *mal*G, *mal*K
Amino acids transport and metabolism	*asp*A, *dap*E, *dap*D *pro*X, *pep*D, *dsd*A, *glt*B, *gab*T
Lipid transport and metabolism	*isp*F, *isp*D, *yfc*X, *tes*A,
Co-enzymes transport and metabolism	*pdx*J, *yie*E
Inorganic ion transport and metabolism	*cys*I, *fie*F
Nucleotide transport and metabolism	*upp*
Cell wall/ membrane/ envelope biogenesis	*yig*M, *ynf*C, *lpx*C, *mdo*H, *mdo*B, *yec*B
Transcription	*vac*B
Intracellular trafficking, secretion, and vesicular transport	*stb*C, *yid*C
Posttranslational modification, protein turnover, chaperones	*pfl*C, *ptr*, *yhf*A, *phn*U
Replication, recombination and repair.	*yhh*F, *pri*A, *rec*A
Ribosomal structure	*mia*B
Defense mechanisms	*ybh*F, *amp*E
Motility	*ygg*R

Table S2. List of putative *det* genes identified by screening of ordered-mutations' library.

Gene Symbol	Product	General Function
STM1665	putative cytoplasmic protein	Unknown
STM0860	putative inner membrane protein (H+/gluconate symporter and related permeases)	Carbohydrate transport and metabolism
STM1328	putative outer membrane protein	Unknown
STM0272	putative ATPase with chaperone activity; homologue of Yersinia clpB	Posttranslational modification,protein turnover, chaperones
STM3253	putative fructose/tagatose biphosphate aldolase	Carbohydrate transport and metabolism
STM1559	putative glycosyl hydrolase	Carbohydrate transport and metabolism
rfbN	LPS side chain defect: rhamnosyl transferase	Carbohydrate transport and metabolism
fliH	flagellar biosynthesis; possible export of flagellar proteins	Motility
rpoS	sigma S (sigma 38) factor of RNA polymerase, major sigmafactor during stationary phase	Transcription
fljB	Flagellar synthesis: phase 2 flagellin (filament structural protein)	Motility
yciE	putative cytoplasmic protein	Unknown
yfdh	putative glycosyltransferase	Cell wall/ membrane/ envelope biogenesis

PhoL	putative phosphate starvation-inducible protein	Signal transduction mechanisms
yahO	putative periplasmic protein	Unknown

Table 3S. Genes differentially expressed during desiccation**.**

Gene Symbol	Product	General Function	log_2 fold-change
kdpB	potassium-transporting ATPase subunit B	Inorganic ion transport and metabolism	2.33
kdpA	potassium-transporting ATPase subunit A	Inorganic ion transport and metabolism	2.22
kdpC	Potassium-transporting ATPase C chain	Inorganic ion transport and metabolism	2.17
ddg	lipid A biosynthesis lauroyl acyltransferase	Lipid transport and metabolism	1.88
rplB	50S ribosomal protein L2	Translation ,ribosomal structure and biogenesis	1.80
slsA	putative inner membrane protein	secondary metabolites biosynthesis, transport and catabolism	1.75
aceA	isocitrate lyase	Energy production and conversion	1.74
glpK	glycerol kinase	Energy production and conversion	1.68
rplE	50S ribosomal protein L5	Translation ,ribosomal structure and biogenesis	1.66
iscA	iron-sulfur cluster assembly protein	Unknown	1.65
yggN	putative periplasmic protein	Unknown	1.61
dnaK	molecular chaperone DnaK	Posttranslational modification, protein turnover, chaperones. Stress response	1.61
argB	acetylglutamate kinase	Amino acids transport and metabolism	1.60
rplP	50S ribosomal protein L16	Translation ,ribosomal structure and biogenesis	1.55
aceB	malate synthase	Energy production and conversion	1.54
ftsJ	23S rRNA methyltransferase	Translation ,ribosomal structure and biogenesis	1.53
rplC	50S ribosomal protein L3	Translation ,ribosomal structure and biogenesis	1.51
hflB	ATP-dependent zinc-metallo protease	Posttranslational modification, protein turnover, chaperones. Stress response	1.50
rplD	50S ribosomal protein L4	Translation ,ribosomal structure and biogenesis	1.50
rpsC	30S ribosomal protein S3	Translation ,ribosomal structure and biogenesis	1.46
ibpA	small heat shock protein	Posttranslational modification, protein turnover, chaperones. Stress response	1.46
nifU	NifU-like protein	Energy production and	1.43

		conversion	
aceK	bifunctional isocitrate dehydrogenase kinase/phosphatase protein	Signal transduction mechanisms	1.43
rplX	50S ribosomal protein L24	Translation ,ribosomal structure and biogenesis	1.43
rpsJ	30S ribosomal protein S10	Translation ,ribosomal structure and biogenesis	1.43
deaD	cysteine sulfinate desulfinase	Translation ,ribosomal structure and biogenesis	1.42
hisF	imidazole glycerol phosphate synthase subunit	Amino acids transport and metabolism	1.42
gltD	glutamate synthase small subunit	Amino acids transport and metabolism	1.42
hisC	histidinol-phosphate aminotransferase	Amino acids transport and metabolism	1.42
rplW	50S ribosomal protein L23	Translation ,ribosomal structure and biogenesis	1.39
argG	argininosuccinate synthase	Amino acids transport and metabolism	1.33
mglB	galactose transport protein	Carbohydrate transport and metabolism	1.32
rpoH	RNA polymerase sigma factor	Transcription	1.31
glpC	sn-glycerol-3-phosphate dehydrogenase K-small subunit	Energy production and conversion	1.28
gltB	glutamate synthase large subunit	Amino acids transport and metabolism	1.28
rpsD	30S ribosomal protein S4	Translation ,ribosomal structure and biogenesis	1.26
hisA	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase	Amino acids transport and metabolism	1.26
rpsH	30S ribosomal protein S8	Translation ,ribosomal structure and biogenesis	1.25
rpoB	DNA-directed RNA polymerase beta subunit	Transcription	1.25
rpoC	DNA-directed RNA polymerase beta' subunit	Transcription	1.23
icdA	isocitrate dehydrogenase	Energy production and conversion	1.23
yhgI	putative thioredoxin-like protein	Posttranslational modification, protein turnover, chaperones. Stress response	1.23
hisH	imidazole glycerol phosphate synthase subunit HisH	Amino acids transport and metabolism	1.22
rplK	50S ribosomal protein L11	Translation ,ribosomal structure and biogenesis	1.22
argA	N-acetylglutamate synthase	Amino acids transport and metabolism	1.20
rpsK	30S ribosomal protein S11	Translation ,ribosomal structure and biogenesis	1.19
hisD	histidinol dehydrogenase	Amino acids transport and metabolism	1.18
argC	N-acetyl-gamma-glutamyl-phosphate reductase	Amino acids transport and metabolism	1.17
rpsQ	30S ribosomal protein S17	Translation ,ribosomal structure and biogenesis	1.17
nifS	Cysteine desulfurase	Amino acids transport and metabolism	1.16
hisB	imidazole glycerol-phosphate dehydratase/histidinol phosphatase	Amino acids transport and metabolism	1.16

rpoE	RNA polymerase sigma-70 factor	Transcription	1.15
nlpD	lipoprotein	Cell wall/ membrane/ envelope biogenesis	1.14
hslU	ATP-dependent protease	Posttranslational modification, protein turnover, chaperones. Stress response	1.13
mtlA	mannitol-specific enzyme IIABC component	Carbohydrate transport and metabolism	1.13
prlC	oligopeptidase A	Amino acids transport and metabolism	1.13
glpB	anaerobic glycerol-3-phosphate dehydrogenase subunit B	Amino acids transport and metabolism	1.12
fumA	fumarase A	Energy production and conversion	1.12
rpoA	DNA-directed RNA polymerase alpha subunit	Transcription	1.11
rpsG	30S ribosomal protein S7	Translation ,ribosomal structure and biogenesis	1.11
rplO	50S ribosomal protein L15	Translation ,ribosomal structure and biogenesis	1.10
groEL	chaperonin GroEL	Posttranslational modification, protein turnover, chaperones. Stress response	1.08
yceD	putative metal-binding protein	General function prediction only	1.06
rseA	Sigma factor RpoE negative regulatory protein RseA	Signal transduction mechanisms	1.03
exbB	energy transduction protein	Intracellular trafficking secretion, and vesicular transport	1.03
ygjU	putative dicarboxylate permease	Amino acids transport and metabolism	1.03
rplJ	50S ribosomal protein L10	Translation ,ribosomal structure and biogenesis	1.02
rplA	50S ribosomal protein L1	Translation ,ribosomal structure and biogenesis	1.01
pps	phosphoenolpyruvate synthase	Carbohydrate transport and metabolism	1.00
argE	acetylornithine deacetylase	Amino acids transport and metabolism	0.99
nlpI	lipoprotein	General function prediction only	0.98
sixA	phosphohistidine phosphatase	Signal transduction mechanisms	0.97
phoH	phosphate starvation-inducible protein	Signal transduction mechanisms	0.96
pgk	phosphoglycerate kinase	Carbohydrate transport and metabolism	0.96
ycjX	putative ATPase	General function prediction only	0.95
prpE	putative acetyl-CoA synthetase	Lipid transport and metabolism	0.95
fusA	elongation factor EF-2	Translation ,ribosomal structure and biogenesis	0.95
pckA	phosphoenolpyruvate carboxykinase	Signal transduction mechanisms	0.93
phoL	putative phosphate starvation-inducible protein	Signal transduction mechanisms	0.93

ydaA	universal stress protein UspE.	Signal transduction mechanisms	0.92
glgA	glycogen synthase	Carbohydrate transport and metabolism	0.91
miaA	tRNA delta(2)-isopentenylpyrophosphate transferase	Translation ,ribosomal structure and biogenesis	0.90
minE	cell division topological specificity factor MinE.	Cell cycle control, cell division, chromosome partitioning	0.89
glgP	glycogen phosphorylase	Carbohydrate transport and metabolism	0.89
ybeL	hypothetical protein STY0704.	Unknown	0.89
sufD	cysteine desulfurase modulator	Posttranslational modification, protein turnover, chaperones. Stress response	0.89
fnr	transcriptional regulator	Signal transduction mechanisms	0.88
prpD	2-methylcitrate dehydratase	General function prediction only	0.87
argD	DapATase	Amino acids transport and metabolism	0.87
argI	ornithine carbamoyltransferase	Amino acids transport and metabolism	0.87
greA	transcription elongation factor	Transcription	0.86
STM1731	putative catalase	Inorganic ion transport and metabolism	-0.87
STM_sRNA_tke1	small RNA	small RNA	-0.9
STM_PSLT068	putative ParB-like nuclease	virulence	-0.9
traN	mating pair stabilization protein	virulence	-1.1
parA	plasmid partition protein A	virulence	-1.18
trbH	conjugative transfer protein	General function prediction only	-1.26
cutC	Copper homeostasis protein	Inorganic ion transport and metabolism	-2.04